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Effect of Temperature, pH, and Initial Cell Number on *luxCDABE* and *nah* Gene Expression during Naphthalene and Salicylate Catabolism in the Bioreporter Organism *Pseudomonas putida* RB1353

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One limitation of employing *lux* bioreporters to monitor in situ microbial gene expression in dynamic, laboratory-scale systems is the confounding variability in the luminescent responses. For example, despite careful control of oxygen tension, growth stage, and cell number, luminescence from *Pseudomonas putida* RB1353, a naphthalene-degrading *lux* bioreporter, varied by more than sevenfold during saturated flow column experiments in our laboratory. Therefore, this study was conducted to determine what additional factors influence the luminescent response. Specifically, this study investigated the impact of temperature, pH, and initial cell number (variations within an order of magnitude) on the peak luminescence of *P. putida* RB1353 and the maximum degradation rate (V_{max}) during salicylate and naphthalene catabolism. Statistical analyses based on general linear models indicated that under constant oxygen tension, temperature and pH accounted for 98.1% of the variability in luminescence during salicylate catabolism and 94.2 and 49.5% of the variability in V_{max} during salicylate and naphthalene catabolism, respectively. Temperature, pH, and initial substrate concentration accounted for 99.9% of the variability in luminescence during naphthalene catabolism. Initial cell number, within an order of magnitude, did not have a significant influence on either peak luminescence or V_{max} during salicylate and naphthalene catabolism. Over the ranges of temperature and pH evaluated, peak luminescence varied by more than 4 orders of magnitude. The minimum parameter deviation required to alter *lux* gene expression during salicylate and naphthalene catabolism was a change in temperature of 1°C, a change in pH of 0.2, or a change in initial cell number of 1 order of magnitude. Results from this study indicate that there is a need for careful characterization of the impact of environmental conditions on both the expression of the reporter and catabolic genes and the activities of the gene products. For example, even though *lux* gene expression was occurring at ~35°C, the luciferase enzyme was inactive. Furthermore, this study demonstrates that with careful characterization and standardization of measurement conditions, the attainment of a reproducible luminescent response and an understanding of the response are feasible.

One of the major constraints on implementing in situ bioremediation is the lack of understanding of how physical, biological, and chemical factors affect microbial activity (7). In an attempt to understand how these factors impact bioremediation, reporter organisms have been developed that allow monitoring of microbial interaction with organic compounds in dynamic systems (6, 20). Reporter organisms are genetically engineered organisms in which a reporter gene(s), such as *lux*, *luc*, or *gfp*, that encodes a detectable gene product is under regulatory control of an inducible catabolic operon (12). In general, due to a relatively short half-life, luciferase (*lux* or *luc*) is preferable in applications where dynamic measurement of gene expression is required (3, 5, 12, 21). However, several groups have observed that bioluminescence is sensitive to physiological and environmental factors (2, 4, 8, 10, 15, 17, 19, 22). Therefore, in order to utilize reporter organisms to monitor in situ gene expression in dynamic, laboratory-scale systems, a comprehensive analysis of the parameters influencing the expression of the catabolic operon and the reporter gene(s) must be conducted.

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In *lux* bioreporters, the luminescence reaction is catalyzed by the luciferase enzyme and involves the oxidation of an aliphatic aldehyde and a reduced flavin mononucleotide ($FMNH_2$), with the liberation of energy in the form of blue-green light at 490 nm. The luciferase enzyme is encoded by the *luxAB* genes, and the aliphatic aldehyde is regenerated by a fatty acid reductase complex encoded by the *luxCDE* genes (16). The *lux* reporter organism employed in this study, *Pseudomonas putida* RB1353, was developed by Burlage et al. (4) and contains two plasmids, NAH7 and pUTK9 (kanamycin resistance). Plasmid NAH7 contains genes for naphthalene catabolism that are organized into two operons, referred to as the upper and lower pathways. The upper pathway codes for the breakdown of naphthalene to salicylate, while the lower pathway codes for the breakdown of salicylate to acetylaldehyde and pyruvate (23). The primary inducer for the upper and lower pathways is salicylate. Plasmid pUTK9 is a genetic construct and contains a subclone in which the NAH7 upper pathway promoter is fused with the *luxCD ABE* genes from *Vibrio fischeri* (4). When exposed to naphthalene, *P. putida* RB1353 exhibits a luminescent response that correlates with naphthalene catabolism (4).

In this study, the main substrate of interest was naphthalene, a polycyclic aromatic hydrocarbon and a U.S. Environmental Protection Agency priority pollutant (11). However, salicylate was also used to characterize expression since salicylate is an

inducer of both the upper and lower pathways on the NAH7 plasmid. Furthermore, salicylate is nonvolatile with relatively high solubility whereas naphthalene is volatile with relatively low solubility (31.7 mg liter^{-1} at 25°C) (1). Therefore, salicylate was used to initially assess how the expression of the *lux* and *nah* genes is affected by temperature, pH, and initial cell number.

By comparison of *P. putida* RB1353 to the parent strain, *P. putida* HK53, Burlage et al. (4) have shown that the *lux* genes do not have an inhibitory effect on the expression of the naphthalene catabolic operon. Furthermore, a previous study examined the influence of parameters such as cell growth stage, initial cell number (order of magnitude variations), substrate concentration, oxygen tension, and the interference of potential background carbon substrates on *P. putida* RB1353 *nah* and *lux* gene expression during salicylate catabolism (17). However, despite control of these parameters, large variations in bioluminescence between repeated experiments were observed in our laboratory when *P. putida* RB1353 was employed to study in situ gene expression during salicylate catabolism in a saturated soil flow column (data not published). Therefore, elucidation of the impact of additional parameters, such as temperature, pH, and initial cell number (variations within an order of magnitude), on the expression of the *lux* genes and the naphthalene and salicylate catabolic operons was warranted. Furthermore, resolving the sensitivity of the luminescent response to these parameters was determined to be essential to enhance the detection of *P. putida* RB1353 gene expression in soil systems. In soil, luminescence is attenuated by a factor of 10 or more depending on the soil employed. Therefore, the objective of this research was to elucidate the sensitivity and improve the reproducibility of the luminescent response of *P. putida* RB1353 to augment the utility of *P. putida* RB1353 as an indicator of *nah* gene expression under dynamic conditions in soil systems.

MATERIALS AND METHODS

Bacterial strain and growth media. *P. putida* RB1353(NAH7, pUTK9) was kindly provided by Robert Burlage, University of Wisconsin, Milwaukee, and Gary Sayler, Center for Environmental Biotechnology, University of Tennessee, Knoxville. Stock solutions of *P. putida* RB1353 were stored in 50% glycerol (Spectrum Chemical Mfg. Corp., New Brunswick, N.J.) at −20°C. To prevent plasmid loss, the stock solutions were used to inoculate a fresh culture for each experiment. Cultures were prepared in Luria broth (LB) containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 1 liter of deionized H_2O. The pH was adjusted to 7.0, and the medium was supplemented with 100 mg of kanamycin sulfate (Sigma Chemical Co., St. Louis, Mo.) liter^{-1} to select for pUTK9. Agar plates were prepared by adding 15 g of Bacto Agar (Difco Laboratories, Detroit, Mich.) liter^{-1} to the LB medium. Mineral salts broth (MSB) was used as the medium for growth on sodium salicylate and naphthalene and contained (in grams per liter) KH_2PO_4 (1.5), Na_2HPO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.2), NH_4Cl (2.5), $FeCl_3$ (3×10^{-4}), and $CaCl_2 \cdot 2H_2O$ (0.013; Spectrum Chemical Mfg. Corp.).

Cell preparation. The following procedure was used to culture cells for all experiments. *P. putida* RB1353 LB streak plates were prepared and maintained at 24°C for 48 h. A single colony was selected and used to prepare a preculture by inoculating 30 ml of LB into a 250-ml Erlenmeyer flask. The flask was placed on a 200-rpm shaker maintained at 24°C. After 24 h, a 100-μl aliquot was withdrawn and serial dilutions were prepared in 0.85% saline. Growth cultures were inoculated by dispensing 100 μl of the 10^7-CFU ml^{-1} dilution into 30 ml of LB in a 250-ml Erlenmeyer flask. The flask was placed on a 200-rpm shaker maintained at 24°C. After the culture reached stationary phase, at approximately 48 h (17), a 29-ml aliquot was withdrawn from the growth culture, centrifuged (centrifuge model J2-21 with JA-20 rotor; Beckman Instruments, Inc., Fullerton, Calif.) for 10 min at 9,800 × *g* to pellet the cells, washed once in 29 ml of 0.85% NaCl, and resuspended in 29 ml of MSB. The final suspension was approximately 10^9 CFU ml^{-1}. Cell density was initially determined spectrophotometrically (550 nm) with a U-2000 spectrophotometer (Hitachi Instruments, Inc., Fremont, Calif.) and later confirmed by viable LB plate counts of serial dilutions.

Quantitation of sodium salicylate, naphthalene, dissolved oxygen (DO), temperature, pH, and luminescence. (i) Sodium salicylate. Samples (1 ml) were added to 0.1 ml of 2.75 M NaOH to disrupt the cell membranes and inhibit further sodium salicylate degradation. While waiting to be analyzed, the samples were stored in the dark to prevent photolysis of sodium salicylate. Before analysis, the samples were centrifuged (Eppendorf model 5415C centrifuge) at 16,000 × *g* for 10 min to pellet cell debris. The sample supernatants were analyzed for sodium salicylate concentration on a U-2000 spectrophotometer at 296 nm, and the concentrations were plotted as a function of sampling time. Sodium salicylate concentrations were determined from a standard curve.

(ii) Naphthalene. Samples (10 ml) were added to 2 ml of dichloromethane in 16-ml glass chromatography vials and sealed with Teflon-coated septa. The vials were inverted five times to promote partitioning of the naphthalene into the dichloromethane and then stored overnight in the dark. The extraction process separated the cells from the naphthalene, thereby preventing cell interference during naphthalene analysis. Next, 1 ml of dichloromethane was withdrawn from each sample and injected into a 2-ml glass chromatography vial sealed with a Teflon septum. The samples were then loaded onto a GC-17A gas chromatograph (Shimadzu, Columbia, Md.) and analyzed for naphthalene by using an RTX-5 chromatography column (length, 15 m; inside diameter, 0.25 mm; film thickness, 0.25 μm; Resteck Corp., Bellefonte, Pa.). The carrier gas was helium at 1.2 ml min^{-1}, measured at 65°C. The oven program was 65°C for 1 min, 65 to 210°C at 15°C min^{-1}, and then 210°C for 3 min. The injector was splitless at 200°C, and the detector was a flame ionization detector at 210°C with helium as the makeup gas. The naphthalene concentrations were determined by comparing the areas under the naphthalene peaks to a standard curve and plotted as a function of the sampling time.

(iii) DO. Samples (1 ml) were analyzed for DO with a micro-oxygen electrode and an oxygen meter (Microelectrodes, Inc., Bedford, N.H.). For each sample, the amount of DO was measured for 1 min immediately following sample collection. The oxygen meter was calibrated by using buffer sparged with N_2, ambient air, and O_2 gas for values of 0, 8.5, and 40 mg liter^{-1}, respectively. In all experiments, the amount of DO never decreased below 8.5 mg liter^{-1}.

(iv) Temperature. Samples (5 ml) were analyzed for temperature immediately after collection in 20-ml glass vials by using a chemical thermometer. The thermometer was calibrated to a digital thermostat and has an accuracy of ±0.1°C.

(v) pH. Analysis of pH was performed on the temperature samples immediately after collection by using a hydrogen electrode and a Beckman Φ34 pH meter. The pH meter was calibrated with pH 4 and pH 7 standards and has an accuracy of ±0.01.

(vi) Luminescence. Samples (2 ml) were analyzed for luminescence in 20-ml glass scintillation vials by using a 1600TR Tri-Carb liquid scintillation analyzer (Packard Instrument Co., Meriden, Conn.). Samples were immediately counted for 1 min in the single-photon mode, generating relative values expressed in counts per minute. The luminescence values obtained were plotted as a function of time and compared to values expressing substrate degradation.

Temperature, pH, and initial cell number experiments. Ten series of experiments were conducted to evaluate the effects of temperature, pH, and initial cell number on *P. putida* RB1353 *lux* and *nah* gene expression. The order and parameters of the experiments were determined based on a stepwise protocol, and cell growth stage, substrate concentration, and DO were controlled according to Neilson et al. (17). Salicylate, which is relatively easy to use compared to naphthalene, was employed initially to assess how *lux* and *nah* gene expression is affected by temperature, pH, and initial cell number. First, a series of temperature experiments was conducted at a specific pH (~7) and cell number (~5×10^7 CFU ml^{-1}) to determine the sensitivity of *lux* gene expression (i.e., peak luminescence) to temperature. Next, a series of pH experiments was conducted at ~24°C and ~5×10^7 CFU ml^{-1} to determine the sensitivity of *lux* gene expression to pH. Finally, a series of initial cell number experiments was conducted at ~24°C and pH ~6.2 to determine the sensitivity of *lux* gene expression to initial cell number. At the start of each experiment, samples (1 ml) were withdrawn and serial dilutions were plated onto LB plates to determine the initial cell number in each experimental culture. During the experiments, samples were withdrawn from each culture every 15 to 20 min and analyzed for salicylate or naphthalene, DO, pH, temperature, and luminescence as described above.

(i) Salicylate: temperature and pH experiments. Cultures were prepared by adding 20 ml of cells, prepared as described above, and 20 ml of filter-sterilized sodium salicylate (1,000 mg liter^{-1}) to 1.96 liters of MSB in a 2.8-liter Erlen-

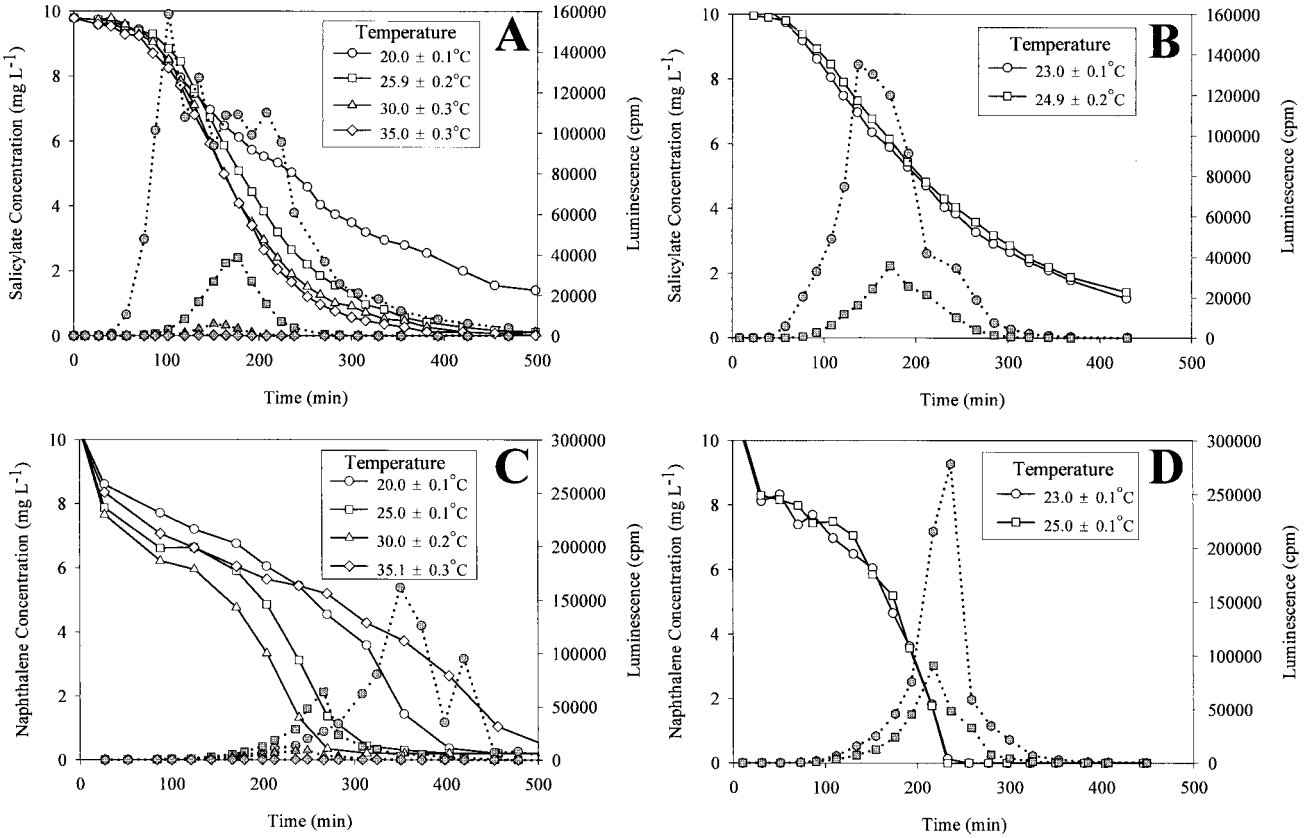

FIG. 1. *P. putida* RB1353 substrate degradation and luminescence over a range of temperatures during salicylate (A and B) and naphthalene (C and D) catabolism. Solid lines with open symbols represent substrate degradation, and dotted lines with closed symbols represent luminescence.

meyer flask. After the contents of the flask were mixed, 300 ml was dispensed into 1-liter Erlenmeyer flasks; the number of flasks was contingent upon how many different temperatures or pHs were being evaluated. For the temperature experiments, the flasks were incubated in environmental shakers (Orbit-Environ shaker, model 3527; Lab-Line Instruments Inc., Melrose Park, Ill.) maintained at the desired temperature and 200 rpm. For the pH experiments, the flasks were incubated in shakers maintained at ~24°C and 200 rpm.

(ii) Salicylate: initial cell number experiments. Cultures were prepared by adding the appropriate volume of cells, prepared as described above, and 3 ml of filter-sterilized sodium salicylate (1,000 mg liter^{-1}) to MSB in each of four 1-liter Erlenmeyer flasks (total volume per flask, 300 ml). The flasks were incubated in shakers (Orbit-Environ shaker, model 3527; Lab-Line Instruments Inc.) maintained at 24°C, 200 rpm, and pH 6.2.

(iii) Naphthalene: temperature, pH, and initial cell number experiments. Parameters for the naphthalene experiments were selected based on the results of the salicylate experiments. One difference is that all salicylate experiments were conducted in 1-liter glass Erlenmeyer flasks whereas all naphthalene experiments were conducted in custom-made 3-liter Tedlar bags (Midan Co., Chino, Calif.) with zero headspace. However, control experiments were performed with salicylate in Tedlar bags to ensure that the difference in experimental conditions for salicylate and naphthalene cultures did not influence *P. putida* RB1353 luminescence or substrate degradation. Each Tedlar bag contained a gas evacuation valve, a septum-covered port for sample extraction, a valved port for fluid injection, and a glass stir bar for mixing. Tedlar bags were filled by using an Acuflow Series II pump (Fisher Scientific International Inc., Pittsburg, Pa.) at a flow rate of 8 ml min^{-1}. During pumping, a saturated naphthalene solution (30 mg of naphthalene liter^{-1} of MSB), prepared by adding naphthalene crystals to 1 liter of sterile MSB and mixing for 24 h, was filtered through a stainless steel glass wool filter to remove any remaining naphthalene crystals and diluted with oxygen-sparged MSB to obtain the desired naphthalene concentration. Oxygen sparging of the MSB solution was necessary to prevent O_2 levels in the cultures from dropping below 8.5 mg of O_2 liter^{-1} during naphthalene degradation. All samples were withdrawn with glass syringes and stainless steel needles to prevent naphthalene volatilization and sorption. For all experiments, the total volume in each Tedlar bag was 1.8 liters. After the bags were filled with the appropriate volume of naphthalene-MSB solution, the desired amount of cells, prepared as described above, was injected through the Teflon septum port and the contents of all Tedlar bags were mixed by using stir plates. For the temperature experiments, the Tedlar bags were placed in environmental shakers set to the desired temperature. For the pH experiments, the pH was adjusted to the desired value prior to cell injection by using 1 M NaOH or 1 M HCl.

Influence of substrate on peak luminescence. A series of experiments with *P. putida* RB1353 was conducted to evaluate the influence of substrate type on peak luminescence. Both salicylate and naphthalene experiments were conducted in Tedlar bags, filled as described above. All experiments were conducted by using the same experimental conditions (i.e., substrate concentration of 6.25×10^{-5} M, 23.5°C, pH 6.15, ~5×10^7 CFU ml^{-1}, 30 mg of DO liter^{-1}, mixing rate of 100 rpm). At the start of each experiment, samples (1 ml) were withdrawn and serial dilutions were plated onto LB plates to determine the initial cell number in each experimental culture. During the experiments, samples were withdrawn from each culture every 15 to 20 min and analyzed for salicylate or naphthalene, DO, pH, temperature, and luminescence as described above.

Statistical analysis. To evaluate the significance of three parameters, temperature, pH, and initial cell number, on *P. putida* RB1353 luminescence, SYSTAT (version 9; SYSTAT Software Inc., Richmond, Calif.) was used to develop general linear models. Specifically, each parameter was evaluated to determine if a relationship existed between the parameter and the luminescent response. Furthermore, the interactions between parameters were evaluated to determine whether parameter interactions were significantly influencing peak luminescence. In all cases, the independent variables were temperature, pH, initial cell number, or initial substrate concentration and the dependent variable was luminescence. Initial substrate concentration was included since some variability in initial substrate concentrations existed (9.78 ± 0.25 mg liter^{-1} for salicylate and 10.25 ± 0.1 mg liter^{-1} for naphthalene). To determine whether interactions between independent variables were influencing peak luminescence on a log basis, the $\log_{10}$ and $\log_e$ of luminescence were calculated and used as the dependent variable. Furthermore, the $\log_{10}$ and $\log_e$ of the independent variables were calculated and used to evaluate whether interactions between the indepen-

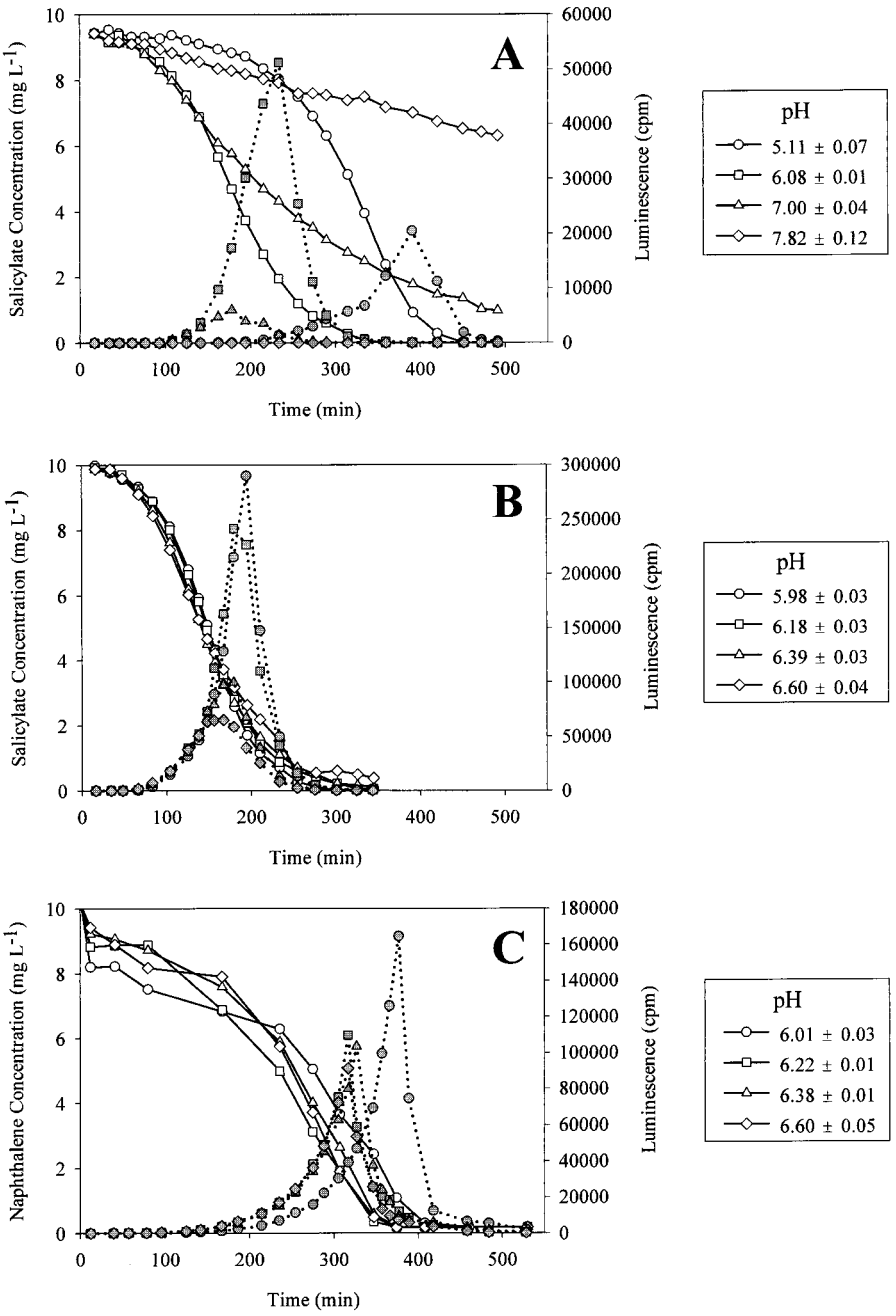

FIG. 2. *P. putida* RB1353 substrate degradation and bioluminescence over a range of pHs during salicylate (A and B) and naphthalene (C) catabolism. Solid lines with open symbols represent substrate degradation, and dotted lines with closed symbols represent luminescence.

dent variables were occurring on a sigmoidal basis. In addition, maximum degradation rates (V_{max}) for substrates were calculated from the slopes of the substrate concentration curves and used as the dependent variable to determine what independent variables were influencing V_{max}.

RESULTS

Temperature experiments. From the series of experiments conducted to determine the impact of temperature on the luminescent response of *P. putida* RB1353 and on substrate degradation, temperature was found to have a significant influence during both salicylate catabolism and naphthalene catabolism (Fig. 1). Over the temperature range tested during salicylate catabolism, peak luminescence varied over 3 orders of magnitude (272 to 158,536 cpm) as temperature decreased from 35 to 20°C (Fig. 1A). V_{max} varied 2.05-fold (1.41 mg h^{-1} [$R^2 = 0.984$; $n = 14$] to 2.90 mg h^{-1} [$R^2 = 0.996$; $n = 9$]), with V_{max} lowest at 20°C and approximately the same at 25, 30, and 35°C. While peak luminescence occurred at 20.0 ± 0.1°C, the luminescent response was erratic and oscillated during salicylate catabolism. Furthermore, V_{max} was significantly lower at 20.0 ± 0.1°C (1.41 mg h^{-1} [$R^2 = 0.984$; $n = 14$]) than at 25.9 ± 0.2°C (2.90 mg h^{-1} [$R^2 = 0.996$; $n = 9$]). Therefore, a second

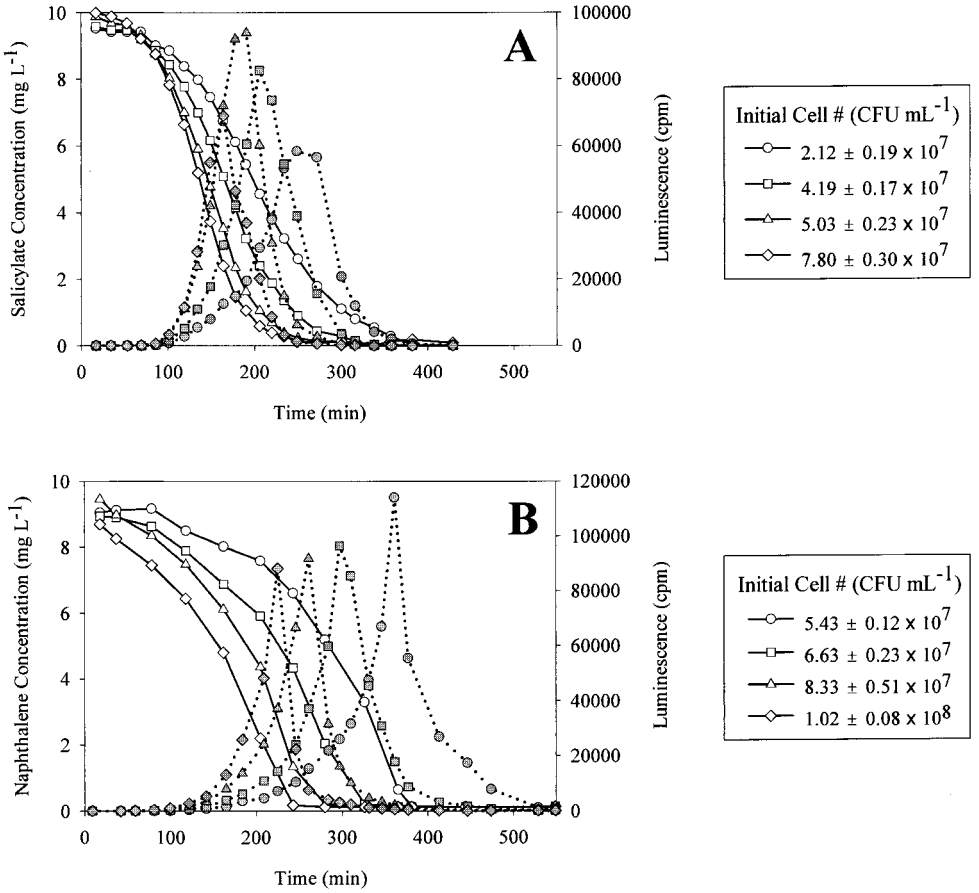

FIG. 3. *P. putida* RB1353 substrate degradation and bioluminescence over a range of initial cell numbers during salicylate (A) and naphthalene (B) catabolism. Solid lines with open symbols represent substrate degradation, and dotted lines with closed symbols represent luminescence.

experiment was performed to investigate the impact of temperatures between 20 and 25°C on the luminescent response. Results showed a peak luminescence of 135,216 cpm and a V_{max} of 2.00 mg h^{-1} ($R^2 = 0.997$; $n = 9$) at 23°C (Fig. 1B).

Over the range of temperatures tested during naphthalene catabolism (20 to 35°C), peak luminescence varied over 3 orders of magnitude (480 to 278,256 cpm) and V_{max} varied 3.94-fold (1.34 mg h^{-1} [$R^2 = 0.984$; $n = 6$] to 5.28 mg h^{-1} [$R^2 = 0.996$; $n = 4$]) (Fig. 1C and D). As temperature increased from 20 to 35°C, luminescence decreased. V_{max} was lowest at 35°C, intermediate at 20°C, and highest at 25 and 30°C. Further testing at 23°C demonstrated a peak luminescence of 278,256 cpm and a V_{max} of 4.60 mg h^{-1} ($R^2 = 0.988$; $n = 4$) (Fig. 1D).

pH experiments. Following the temperature study, a series of experiments was conducted to determine the impact of pH on the luminescent response of *P. putida* RB1353 and on substrate degradation. Both luminescence and substrate degradation were found to be significantly influenced by pH during salicylate and naphthalene catabolism (Fig. 2A and B). Over the pH range tested during salicylate catabolism (pH 5 to 8), peak luminescence varied over 4 orders of magnitude (16 to 290,000 cpm) and V_{max} varied 8.89-fold (0.440 mg h^{-1} [$R^2 = 0.992$; $n = 8$] to 3.89 mg h^{-1} [$R^2 = 0.989$; $n = 9$]). Peak luminescence was higher at pH 6 than at pH 5 and decreased as pH increased from 6 to 8. V_{max} remained relatively constant from pH 5 to pH 6.6 and then decreased as pH increased from 6.6 to 8.

A smaller pH range was evaluated during naphthalene catabolism (pH 6.0 to 6.6) (Fig. 2C). The variations observed in peak luminescence and V_{max} were 1.8-fold and no significant variation, respectively. As pH increased from 6.0 to 6.6, peak luminescence decreased and V_{max} remained relatively constant.

Initial cell number experiments. Following the pH study, a series of experiments was conducted to determine the impact of initial cell number on the luminescent response of *P. putida* RB1353 and on substrate degradation (Fig. 3). Varying initial cell numbers between 2×10^7 CFU ml^{-1} and 8×10^7 CFU ml^{-1} during salicylate catabolism and between 5×10^7 CFU ml^{-1} and 1×10^8 CFU ml^{-1} during naphthalene catabolism did not have a significant impact on peak luminescence or on V_{max}.

Sensitivity and reproducibility of luminescent response. The sensitivity of the luminescent response of *P. putida* RB1353 to temperature, pH, and initial cell number during salicylate and naphthalene catabolism was determined by examining the experimental conditions and peak luminescences for all the experiments conducted. The sensitivities of the luminescent response were found to be similar during both salicylate and naphthalene catabolism. As illustrated by the three-dimen-

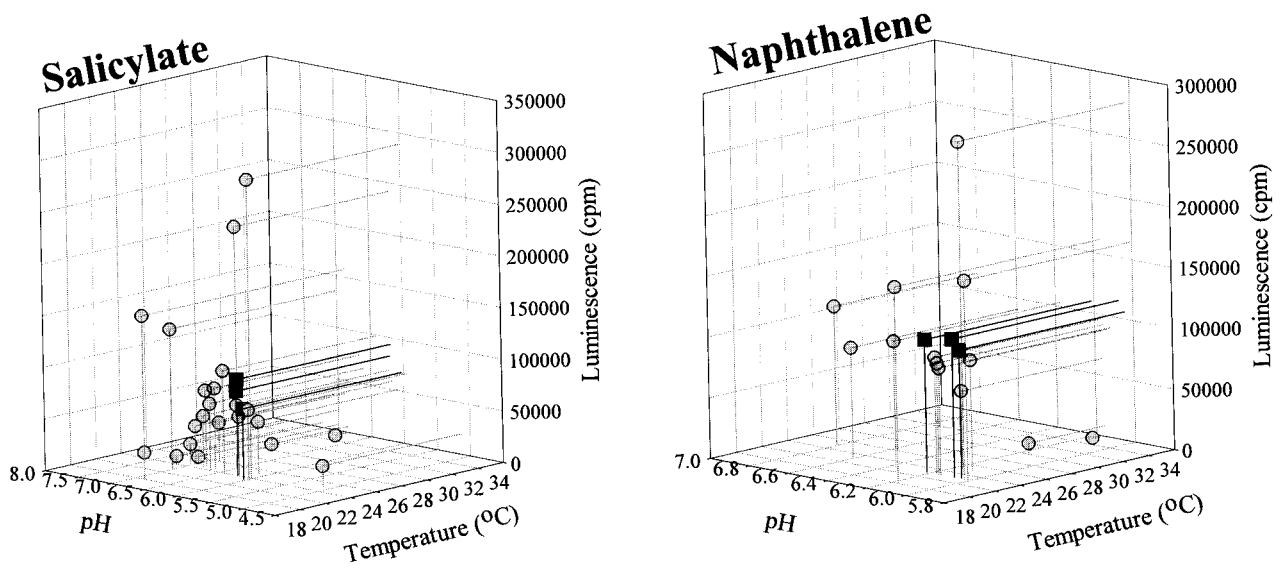

FIG. 4. Three-dimensional plots of temperature, pH, and peak luminescence for all salicylate and naphthalene catabolism experiments. Squares (■) indicate the salicylate and naphthalene experiments that were conducted to evaluate the reproducibility of the luminescent response.

sional plots of temperature, pH, and peak luminescence, the minimum parameter deviation required to alter *lux* gene expression was a change of 1°C in temperature, a change of 0.2 in pH, or a change of 1 order of magnitude in initial cell number (Fig. 4).

To confirm this observation and to determine the reproducibility of the luminescent response, three salicylate and three naphthalene catabolism experiments were conducted in which temperature, pH, and initial cell number were constrained within the minimum deviations indicated by the present study (23.5 ± 0.5°C, pH 6.1 ± 0.1, and $5.0 \times 10^7 \pm 2.5 \times 10^7$ CFU ml^{-1}) and in which cell growth stage, substrate concentration, and DO were controlled according to Neilson et al. (17) (Fig. 4). By dividing the standard deviations by the average peak luminescence values for the three salicylate and three naphthalene experiments, the peak luminescence values were determined to vary by 15.6 and 3.81% for the salicylate and naphthalene catabolism experiments, respectively.

Influence of substrate on peak luminescence. Under the same experimental conditions and substrate concentration, the peak luminescence during salicylate catabolism was greater than the peak luminescence during naphthalene catabolism (data not shown). The difference in peak luminescence may be a result of substrate-induced membrane disruption, which alters the availability of fatty acids for the luminescent reaction (10). Additional research is currently being conducted in our laboratory to further elucidate this phenomenon.

Statistical analysis. During salicylate catabolism, peak luminescence and V_{max} were a function of temperature and pH. As indicated by the R^2 values, the influence of temperature and pH accounted for 98.1% of the variation observed in peak luminescence and 94.2% of the variation observed in V_{max} (Table 1). During naphthalene catabolism, peak luminescence was a function of temperature, pH, and initial naphthalene concentration and V_{max} was a function of temperature and pH. As indicated by the R^2 values, the influence of temperature, pH, and initial substrate concentration accounted for 99.9% of the variation observed in peak luminescence and the influence of temperature and pH accounted for 49.5% of the variation observed in V_{max} (Table 2).

DISCUSSION

When *lux* bioreporters are employed to monitor in situ microbial gene expression in dynamic, laboratory-scale systems, the physiological conditions of the organism as well as environmental conditions must be considered when interpreting the bioluminescent response. Since catabolic *lux* bioreporters

TABLE 1. Factors influencing peak luminescence and V_{max} during salicylate catabolism

Dependent variable	Effect(s) or source	Coefficient	SE	*t*	*P*	Sum of squares	Degrees of freedom	Mean square	*F* ratio
$\log_e$ (luminescence)	Temperature	9.25×10^{-1}	1.55×10^{-1}	5.95	1.00×10^{-5}				
($R^2 = 0.981$; $n = 24$)	pH	2.93	4.42×10^{-1}	6.64	1.40×10^{-6}				
	Temperature × pH	-1.95×10^{-1}	2.67×10^{-2}	−7.32	3.20×10^{-7}				
	Regression				9.99×10^{-16}	2.60×10^{3}	3	8.66×10^{2}	3.69×10^{2}
	Residual					4.92×10^{1}	21	2.34	
V_{max} ($R^2 = 0.942$; $n = 23$)	Temperature	4.83×10^{-1}	7.47×10^{-2}	6.47	2.00×10^{-6}				
	Temperature × pH	-5.67×10^{-2}	1.13×10^{-2}	−5.01	6.00×10^{-5}				
	Regression				1.09×10^{-13}	1.88×10^{2}	2	9.42×10^{1}	1.69×10^{2}
	Residual					1.17×10^{1}	21	5.55×10^{-1}	

TABLE 2. Factors influencing peak luminescence and V_{max} during naphthalene catabolism

Dependent variable	Effect(s) or source[a]	Coefficient	SE	*t*	*P*	Sum of squares	Degrees of freedom	Mean square	*F* ratio
$\log_e$ (luminescence)	Temperature	-2.39×10^1	7.11	−3.36	7.22×10^{-3}				
($R^2 = 0.999$; $n = 16$)	pH	5.91×10^1	2.18×10^1	2.72	2.18×10^{-2}				
	C_0[a]	2.44×10^1	7.86	3.11	1.12×10^{-2}				
	Temperature × pH	1.53	5.37×10^{-1}	2.86	1.71×10^{-2}				
	Temperature × C_0	1.37	5.32×10^{-1}	2.58	2.74×10^{-2}				
	pH × C_0	−9.40	2.79	−3.37	7.18×10^{-3}				
	Regression				4.33×10^{-15}	2.01×10^3	6	3.35×10^2	2.28×10^3
	Residual					1.47	10	1.47×10^{-1}	
V_{max} ($R^2 = 0.495$; $n = 15$)	Constant	-3.09×10^3	1.05×10^3	−2.93	1.37×10^{-2}				
	Temperature	1.34×10^2	4.57×10^1	2.94	1.34×10^{-1}				
	pH	5.00×10^2	1.71×10^2	2.93	1.36×10^{-2}				
	Temperature × pH	-2.18×10^1	7.42	−2.94	1.34×10^{-2}				
	Regression				4.99×10^{-2}	6.82	3	2.27	3.59
	Residual					6.97	11	6.34×10^{-1}	

[a] C_0, Initial substrate concentration. Constant, predicted value of the dependant variable when all regressors are set to zero.

are constructed based on transcriptional fusions between *lux* genes and promoters from hydrocarbon degradation pathways, both the *lux* genes and the catabolic genes are expressed simultaneously. However, this study indicates that the activities of the *lux* and catabolic gene products may be impacted in different ways when subjected to the same environmental and physiological conditions. For example, during salicylate catabolism from 20 to 35°C, peak luminescence was highest and V_{max} was lowest at 20°C. In contrast, during salicylate and naphthalene catabolism at approximately 35°C, the catabolic gene activity was sufficient to metabolize all of the available substrate but the luciferase activity was virtually zero (i.e., less than 400 cpm) (Fig. 1). The thermostabilities of bacterial luciferases are known to vary depending on the organism in which luciferase is expressed (13). Furthermore, *V. fischeri* luciferase is thermolabile, with enzyme denaturation occurring above 30°C (14). In addition, a similar result was observed during naphthalene catabolism since peak luminescence was highest at pH 6.0, a value below the pH of 7.0 that is optimum for naphthalene dioxygenase activity (Fig. 2) (18). Therefore, even though similar levels of gene expression are occurring for the reporter and catabolic genes, the bacterial luciferase and the catabolic gene product activities exhibit different temperature and pH optima. This suggests that there is a need for careful characterization of the impact of environmental conditions on both the expression of the reporter and catabolic genes and the activities of the gene products in order to understand and optimize in situ reporter performance.

One limitation of employing *lux* bioreporters to monitor in situ microbial gene expression in dynamic, laboratory-scale systems is the confounding variability in the luminescent responses. Several studies indicate that linear luminescent responses are attainable over a range of substrate concentrations when *lux* bioreporters are used as biosensors to determine the bioavailability of environmental pollutants (8, 9). However, these studies do not address the complexity of monitoring the dynamics of in situ gene expression during substrate catabolism (17). The statistical analyses in this study indicate that pH and temperature, in conjunction with the controlled variables of cell growth stage, substrate concentration, and DO levels, account for essentially all the factors affecting the luminescent response in *P. putida* RB1353. With vigilant control over the variables influencing peak luminescence, reproducibility was attained during both salicylate and naphthalene catabolism (Fig. 4).

The results of this investigation demonstrate that after an extensive evaluation of the environmental and physiological parameters influencing *lux* reporter organisms, the attainment of a reproducible luminescent response and an understanding of the response are feasible. Furthermore, the characterization of *P. putida* RB1353 provides a sound basis for understanding how environmental and physiological factors may influence the bioluminescent responses in other *lux* reporter organisms implemented in laboratory-scale systems. However, results from this investigation also suggest that while *lux* bioreporters have tremendous potential for studies of in situ gene expression with simplified, controlled laboratory systems, the interpretation of luminescent responses in complex field environments will be difficult.

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